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JOURNAL OF MEMBRANE BIOLOGY, (1999 Mar 1) 168 (1) 77-89.

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Thank-You!

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Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element

(hemodynamics/atherosclerosis/gene regulation/vascular endothelium)

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ABSTRACT The endothelial lining of blood vessels is constantly exposed to fluid mechanical forces generated by flowing blood. *In vitro* application of fluid shear stresses to cultured endothelial cells influences the expression of multiple genes, as reflected by changes in their steady-state mRNA levels. We have utilized the B chain of platelet-derived growth factor (PDGF-B) as a model to investigate the mechanisms of shear-stress-induced gene regulation in cultured bovine aortic endothelial cells (BAECs). Northern blot analysis revealed elevated endogenous PDGF-B transcript levels in BAECs, after exposure to a physiological level of laminar shear stress (10 dynes/cm²; 1 dyne = 100 mN) for 4 h. A transfected reporter gene, consisting of a 1.3-kb fragment of the human PDGF-B promoter coupled to chloramphenicol acetyltransferase (CAT), indicated a direct effect on transcriptional activity. Transfection of a series of PDGF-B-CAT deletion mutants led to the characterization of a cis-acting component within the PDGF-B promoter that was necessary for shear-stress responsiveness. In gel-shift assays, overlapping oligonucleotide probes of this region formed several protein-DNA complexes with nuclear extracts prepared from both static and shear-stressed BAECs. A 12-bp component (CTCTCAGAGACC) was identified that formed a distinct pattern of complexes with nuclear proteins extracted from shear-stressed BAECs. This shear-stress-responsive element does not encode binding sites for any known transcription factor but does contain a core binding sequence (GAGACC), as defined by deletion mutation in gel-shift assays. Interestingly, this putative transcription factor binding site is also present in the promoters of certain other endothelial genes, including tissue plasminogen activator, intercellular adhesion molecule 1, and transforming growth factor β 1, that also are induced by shear stress. Thus, the expression of PDGF-B and other pathophysiologically relevant genes in vascular endothelium appears to be regulated, in part, by shear-stress-induced transcription factors interacting with a common promoter element.

lium ("endothelial dysfunction") may constitute a pathogenic risk factor for vascular diseases such as atherosclerosis (2).

In addition to local or systemic humoral stimuli, such as cytokines and bacterial products (3), it is now recognized that hemodynamic forces also can influence endothelial structure and function (4–8). In particular, wall shear stress, the tractive force imparted to the vascular lining by flowing blood, can have acute (seconds to minutes) or chronic (hours to days) biological effects (8). Certain of these (e.g., prostacyclin generation) appear to involve the activation of constitutive stimulus-response effector mechanisms (9), whereas others [e.g., tissue plasminogen activator (tPA) production] reflect *de novo* protein synthesis and secretion (10). Recently, based on the measurement of steady-state levels of mRNA, physiological levels of laminar shear stress have been demonstrated to upregulate the expression of several pathophysiologically relevant genes in cultured endothelial cells, including tPA (10), TGF- β 1 (11), and PDGF-A and -B chains (12). However, the cellular and molecular mechanisms underlying this shear-stress responsiveness of vascular endothelium remain incompletely understood.

In this report, we have utilized a well-defined *in vitro* mechanical system and the PDGF-B gene as a model to demonstrate a direct effect of shear stress on gene transcription in vascular endothelial cells. By analyzing the activity of a series of mutant PDGF-B promoter-chloramphenicol acetyltransferase (CAT) reporter gene constructs transfected into cultured bovine aortic endothelial cells (BAECs), we have identified a cis-acting element in the PDGF-B gene that is required for shear-stress responsiveness [shear-stress-responsive element (SSRE)]. A core binding sequence within this element (GAGACC) appears to bind to transcription factors present in the nuclear extracts of shear-stressed BAECs. Interestingly, this sequence is also present in the promoters of certain other endothelial genes that have been found to be up-regulated by shear stress, including tPA, intercellular adhesion molecule 1 (ICAM-1), and TGF- β 1, thus suggesting a general mechanism for induction of gene transcription by this biomechanical stimulus.

MATERIALS AND METHODS

Cell Culture. A BAEC line (11-BAEC) that forms uniformly confluent density-inhibited monolayers with little tendency for "sprout" formation was cultured in Dulbecco's

Abbreviations: PDGF, platelet-derived growth factor; BAEC, bovine aortic endothelial cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; SSRE, shear-stress-responsive element; TGF- β 1, transforming growth factor β 1; ICAM-1, intercellular adhesion molecule 1; tPA, tissue plasminogen activator.

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modified Eagle's medium (GIBCO/BRL) supplemented with 10% (vol/vol) calf serum (Whittaker Bioproducts), 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μ g/ml) as described (6).

Shear-Stress Apparatus. The modified cone-and-plate apparatus utilized for generating defined fluid shear stresses has been described (13, 14). It consists of a stainless steel cone rotating over a base plate that contains 12 1-cm (diameter) plastic coverslip inserts or a single 17-cm (diameter) plastic plate specially fabricated by Costar. Culture medium in the base-plate reservoir (15 ml, total volume) is gradually exchanged (0.5 ml/min) without recirculation. The entire apparatus is maintained in a 5% CO₂/95% air humidified atmosphere and is thermostatically regulated at 37°C. Fluid mechanical parameters (medium viscosity, cone angle, and rotational speed) were adjusted to subject the endothelial monolayers to a uniform laminar shear stress of 10 dynes/cm² (1 dyne = 100 mN) for 4 h. Replicate-plated control coverslips were incubated under static conditions for the same time interval. Shear-stressed and static endothelial monolayers appeared morphologically indistinguishable after 4 h, consistent with previous observations (15).

Recombinant DNA Constructs and Probes. A PDGF-B-CAT reporter gene was constructed from a 1.3-kb fragment of the human PDGF-B chain promoter (16) cloned into an *Xba* I site of a pSP65 backbone carrying the CAT gene in the *Hind*III-*Pvu* II sites. This reporter gene and the endogenous PDGF-B gene show a similar pattern of expression *in vivo* in transgenic mice (16). A series of nested 5'-deletion mutations were carried out within the PDGF-B-CAT construct. A cytomegalovirus-CAT reporter gene construct was kindly provided by S. Orkin (Children's Hospital Medical Center, Boston, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was obtained from the American Type Culture Collection (Rockville, MD) (deposited by R. Wu), and the 0.96-kb *Hind*II-*Bgl* I fragment was used for labeling reactions.

Northern Blot Analysis. Total cellular RNA was extracted from experimental BAEC monolayers, using the SDS/NaOH lysis method (17). Aliquots (5 μ g) of RNA were loaded in each lane, electrophoresed through a 1.2% formaldehyde/agarose gel for 4 h, and stained with ethidium bromide to verify the quality and the quantity of the RNA, prior to blotting and hybridization with ³²P-labeled cDNA probes.

Transfection Procedures and CAT Assay. BAECs were transfected by the modified calcium phosphate procedure (17) and either subjected to shear stress or incubated under static conditions. At the end of the experiment (\approx 50 h after transfection), the cells were extracted, and CAT activity was measured in a liquid assay (17). The transfection efficiency of the various reporter gene constructs was evaluated by extracting transfected BAECs, using the Hirt procedure (18), and transforming circular plasmid DNA to *Escherichia coli* DH5 α , which were plated on ampicillin-containing plates. The number of ampicillin-resistant colonies was taken as a direct index of the efficiency of transfection and used to normalize CAT activities among various experiments.

Extraction of Nuclear Proteins. For the study of DNA binding factors, control (static) or shear-stressed BAEC monolayers were washed with phosphate-buffered saline (calcium/magnesium-free) and trypsinized, and nuclei were extracted as described by Osborn *et al.* (19). Nuclear extracts were kept frozen in liquid N₂ until analysis.

Gel-Retardation Assays. Oligonucleotide probes (30–35 bp) were synthesized in an Applied Biosystems apparatus (model 381A). The binding of the oligonucleotides SS-1, SS-2, and SS-4 was carried out in the presence of 50 mM NaCl/10% (vol/vol) glycerol/0.02% Nonidet P-40, using reagents supplied with the Pharmacia BandShift kit. Each binding experiment included 5–10 μ g of nuclear extracts and 1 pM end-

labeled oligonucleotide, which were incubated at 20°C for 20 min. Binding conditions for the various oligonucleotides were determined in preliminary experiments. The binding of SS-1, SS-2, and SS-4 was maximal in the presence of 50 mM NaCl, whereas SS-3 bound in the presence of either 50 mM NaCl or SP-1 binding buffer (25 mM Tris Cl, pH 7.9/6.25 mM MgCl₂/0.5 mM EDTA/50 mM KCl/0.5 mM dithiothreitol/10% glycerol). Oligonucleotides encoding for SP-1 and Oct-1 binding sites were purchased from GIBCO/BRL. The protein-DNA complexes were electrophoresed through 5% polyacrylamide gels in the presence of 1 \times Tris borate buffer (89 mM Tris base/89 mM boric acid, pH 8.0), dried, and autoradiographed on Kodak x-ray AR films.

RESULTS

Laminar Shear Stress Affects PDGF-B Transcript Levels in Cultured Endothelial Cells. Cultured BAEC monolayers were either exposed to laminar shear stress (10 dynes/cm²) for 4 h in the cone-plate apparatus or incubated under static conditions for the same period of time. Northern blot analysis of RNA revealed that monolayers exposed to shear stress contained elevated levels of endogenous PDGF-B transcripts (Fig. 1). A commonly used "control" gene, GAPDH, interestingly appeared to be down-regulated by shear stress, consistent with recent observations by others in both bovine and human endothelial cells.

Direct Effect of Shear Stress on PDGF-B Transcript in Cultured Endothelial Cells. Increased levels of PDGF-B transcripts under shear stress could result from either stabilization of mRNA or an enhanced rate of transcription (or a combination of both). To investigate whether shear stress can directly influence transcriptional regulation, we constructed a vector carrying a CAT reporter gene regulated by a 1.3-kb fragment of the human PDGF-B promoter (Fig. 2). Cultured BAECs were transfected with this construct and, 24 h later, either exposed to laminar shear stress (10 dynes/cm²) for 4 h or incubated under static conditions for the same period of time. As shown in Table 1, CAT activity derived from the construct carrying the intact PDGF-B promoter was severalfold higher in transfected cells exposed to shear stress. In contrast, similar CAT activity was observed in BAECs under both static and shear-stress conditions, after transfection of a cytomegalovirus reporter gene.

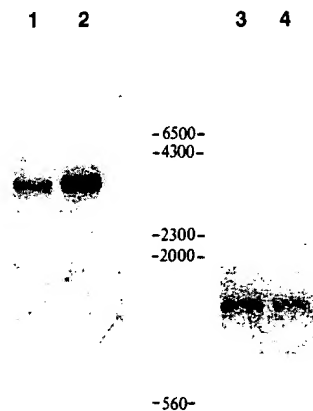


FIG. 1. Effect of laminar shear stress on steady-state levels of PDGF-B chain and GAPDH transcripts in cultured endothelial monolayers. Northern blot analysis of total RNA (5 μ g) extracted from BAECs that had been exposed to laminar shear stress (10 dynes/cm² for 4 h; lanes 2 and 4) or incubated under static conditions (lanes 1 and 3) and hybridized to either ³²P-labeled PDGF-B (lanes 1 and 2) or GAPDH (lanes 3 and 4) probes. Molecular sizes in base pairs are indicated.

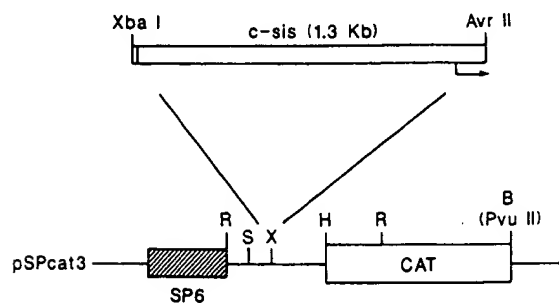


Fig. 2. CAT reporter gene regulated by a 1.3-kb fragment of the human PDGF-B chain promoter.

Definition of a SSRE Within the PDGF-B Promoter. Increased transcription of CAT under shear stress in BAECs transfected with the PDGF-B-CAT construct suggested that there are regions within the PDGF-B promoter that are shear-stress-responsive. To define these element(s), a series of nested 5'-deletion mutations of the PDGF-B promoter coupled to CAT were transfected into BAECs, which then were either exposed to laminar shear stress (10 dynes/cm²) for 4 h or incubated under static conditions in parallel. Analysis of the series of deletion mutants depicted in Fig. 3 revealed that construct d17, which encodes only a third of the intact promoter, was still responsive to shear stress. Construct d77 previously had been identified as the minimal element required for the expression of PDGF-B in cultured endothelial cells under static conditions (20, 21). This construct was not responsive to shear stress. Deletion of a 50-bp element, between constructs d26 and d36, abolished the responsiveness to shear stress, without influencing expression under static conditions. We therefore concluded that the putative SSRE in the PDGF-B promoter lies within the region defined by these two mutants.

Shear Stress Induces Transcription Factors That Bind to the SSRE. To better characterize this putative SSRE, we examined the binding of nuclear proteins extracted from static and shear-stressed BAECs to the region of the PDGF-B promoter delimited by the d26 and d36 mutants, by using gel-shift (mobility retardation) analysis. Three oligonucleotides, encoding the 5', middle, and 3' portions of this region, were synthesized and termed SS-1, SS-2, and SS-3, respectively (Fig. 4). Each oligonucleotide was 25–30 bp long and contained a 12-bp overlapping stretch at their ends. SS-3 was the only oligonucleotide that contained a consensus binding site (GGGCGG) for a known transcription factor, SP-1 (22).

All three oligonucleotides were able to form multiple complexes with nuclear proteins derived from either static or shear-stressed BAECs (Fig. 4A). The quantity of high molecular weight complexes formed with both SS-1 and SS-2 probes was increased in nuclear extracts from shear-stressed cells. Addition of a 100-fold excess of unlabeled SS-1 or SS-2

essentially abolished this binding, indicating the specificity of the observed shift. In contrast, the intensity and pattern of the complexes formed with SS-3, which contains an SP-1 binding site, were similar under static and shear-stress conditions. Moreover, binding of SS-3 to nuclear proteins from static BAECs could be reduced by the addition of unlabeled SS-3 (Fig. 4A) or SP-1 oligonucleotide (data not shown). The ability of relevant unlabeled probes (Fig. 4A, lanes 4, 8, and 12) to inhibit complex formation was not shared by a ubiquitous transcription factor binding site probe (Oct-1) (data not shown).

Identification of the Binding Site for Shear-Stress-Induced Transcription Factors. Because the gel-shift patterns of SS-1 and SS-2 observed in the presence of shear-stressed nuclear extracts were similar, we hypothesized that the relevant binding site(s) were situated in the overlapping stretch at the 3' end of SS-1 and 5' end of SS-2, respectively (Fig. 4). A concatemirized oligonucleotide termed SS-4 that encodes this overlapping 12-bp (CTCTCAGAGACC) stretch, therefore, was synthesized and analyzed in a gel-shift assay. As with SS-1 and SS-2, the gel mobility of SS-4 was retarded in the presence of nuclear extracts from shear-stressed BAECs. This binding was inhibited by the addition of unlabeled SS-4 (Fig. 4B, lane 4) but not with unlabeled Oct-1 (Fig. 4B, lane 5), indicating specificity of the observed gel shift. Concatemirized mutant SS-4 probes, containing the 5' half (CTCTCA), the 3' half (GAGACC), or a mutated form (CTCTCAAGTCC, substituted bases are underlined) or the wild-type SS-4 oligonucleotide, were also analyzed. A similar pattern of gel shift was obtained with the 3', but not the 5', or mutant SS-4 probe, thus further localizing the relevant core binding sequence (data not shown).

Computer analysis of the 5' flanking regions of several other genes (tPA and TGF- β 1) that have been shown to be upregulated by shear stress in vascular endothelium (10–12) revealed that this core binding sequence (GAGACC) or its complementary sequence (GGTCTC) are also present in each of their promoters (Table 2). Interestingly, within the PDGF-B chain gene, this stretch is fully conserved among the murine, feline, and human promoters, although it is located in a region that is otherwise poorly conserved across species (21, 23).

DISCUSSION

Hemodynamic forces in general, and more specifically fluid shear stresses, have been implicated as modifiers of endothelial cell structure and function *in vivo* (4–8). Changes in cell morphology, growth status, and the production and secretion of various effector molecules have been observed in cultured endothelial cells exposed to defined shear stresses *in vitro* (9–14). Many of these changes are dependent, directly or indirectly, on gene expression. This study demonstrates that a mechanical force, such as shear stress, applied to vascular endothelial cells, can regulate gene expression at the level of transcription. By using deletion analysis and gel-shift assays, a SSRE was defined within the PDGF-B promoter. As shown in Table 2, the SSRE is identical in the murine, feline, and human PDGF-B promoters, although it is located in a region that is otherwise poorly conserved across species (21, 23). Moreover, a 6-bp core binding sequence within the SSRE (GAGACC) that bound nuclear proteins in a same pattern as SSRE is encoded by promoters of several other genes that have also been shown to be regulated by shear stress in vascular endothelium (human, murine, and rodent tPA; human and murine TGF- β 1). Recently, we have found (30) that ICAM-1 is also upregulated by shear stress (laminar; 10 dynes/cm²; 4–48 h) in cultured human vascular endothelial cells, at the levels of protein and mRNA. Interestingly, the SSRE core binding sequence (GAGACC) is encoded

Table 1. Effect of laminar shear stress on the expression of PDGF-B-CAT and CMV-CAT constructs in transfected BAECs

Construct	CAT activity				Fold induction
	cpm $\times 10^{-3}$		Normalized		
	Static	Shear	Static	Shear	
None	1.2 \pm 0.2	1.1 \pm 0.1	ND	ND	—
PDGF-B-CAT	11.8 \pm 0.2	41.2 \pm 0.4	25	87	3.5
CMV-CAT	11.5 \pm 0.5	12.9 \pm 0.9	33	37	1.1

BAECs transfected with the PDGF-B-CAT construct (Fig. 2) were exposed to laminar shear stress (10 dynes/cm² for 4 h) or incubated under static conditions for the same period of time. CAT activity is expressed in cpm and normalized to the number of ampicillin-resistant colonies, as an index of the efficiency of transfection. CMV, cytomegalovirus promoter.

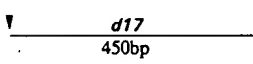
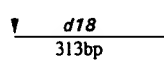



Deletion Construct	CAT Activity				Induction (fold)
	cpm $\times 10^{-3}$ / μ g protein		/Amp colonies		
	static	shear	static	shear	
 d17 450bp	10.2 \pm 0.6	47.4 \pm 0.9	14	63	4.5
 d18 313bp	8.0 \pm 0.2	32.6 \pm 4.9	15	62	4.1
 d26 153bp	9.0 \pm 0.4	37.0 \pm 0.8	25	103	4.1
 d36 101bp	9.5 \pm 0.5	9.3 \pm 0.3	20	19	0.9
 d77 82bp	10.1 \pm 0.3	13.8 \pm 0.3	22	30	1.4

FIG. 3. Deletion analysis of shear-stress responsiveness of the PDGF-B promoter. A series of deletion mutations of the PDGF-B promoter were coupled to CAT, transfected into BAECs, which then were exposed to laminar shear stress (10 dynes/cm² for 4 h) or incubated under static conditions for the same period of time. CAT activity is expressed in cpm/ μ g of protein and as normalized to the number of ampicillin-resistant (Amp) colonies, as an index of the efficiency of transfection and the fold shear-stress induction of CAT activity, is expressed as a ratio (shear/static values).

within the ICAM-1 promoter. In contrast, two other endothelial expressed adhesion molecules, E-selectin and vascular cell adhesion molecule 1, that are not upregulated by the same shear-stress exposure (30) do not contain this core binding sequence in their promoters (32, 33).

Large vessels typically undergo adaptive changes in diameter and wall thickness in response to changes in flow velocities that lead to a redistribution of wall shear stresses (34, 35). A component of this adaptation is very rapid (minutes) and is dependent upon the presence of an intact endothelial lining (36). In preliminary *in vitro* experiments (N.R., unpublished observations), induction of endogenous PDGF-B transcripts was detectable in cultured bovine aortic endothelial monolayers as early as 30 min after initial exposure to laminar shear stress (10 dynes/cm²). These findings agree with recent observations of others (12) on PDGF

expression in human endothelial cells. The acute and more chronic induction of PDGF-B *in vitro* may correlate with the putative actions of this polypeptide *in vivo*, as a vasoconstrictor (37) and as a smooth muscle hyperplastic stimulus (31, 38, 39).

One of the most intriguing questions is the pathway through which externally applied hemodynamic forces signal the nucleus to activate gene transcription. Several studies have demonstrated that shear stress can stimulate inositol phospholipid turnover in cultured endothelial cells, producing second messengers, such as inositol trisphosphate and diacylglyceride, that are involved in the release of calcium from intracellular pools and protein kinase C activation (40–43). Intracellular levels of ionized calcium have been demonstrated to be affected, directly and indirectly, by shear stress in cultured endothelial cells (8, 44). In recent studies, changes in calcium levels and activation of protein kinase C have been implicated in the regulation of PDGF-B chain expression in endothelial cells under both static and flow conditions (45). Conceivably, the cytoskeletal reorganization that occurs in endothelial cells, both *in vitro* and *in vivo*, several hours after exposure to flow (46, 47) may, in conjunction with cell shape change, play a role in gene expression. In addition, several known transcriptional factors appear to be affected by shear stress (48), although their consensus binding sequences are not present in the SSRE defined in this study. In preliminary experiments, changes have been observed (N.R., unpublished observations) in gel-shift patterns with nuclear extracts from shear-stressed BAECs (vs. static controls) by using probes for transcription factors NF- κ B and AP-1, *myc*, and the cAMP responsive

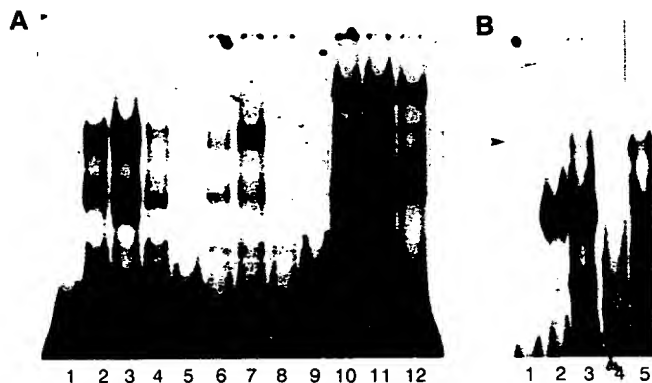


FIG. 4. Binding of nuclear proteins from static and shear-stressed BAECs to the shear-stress-responsive region in the PDGF-B promoter. (A) Gel-shift analysis of the complexes formed by SS-1 (lanes 1–4), SS-2 (lanes 5–8), and SS-3 (lanes 9–12) oligonucleotide probes of the 50-bp shear-stress-responsive region of the PDGF-B promoter with nuclear extracts from static or shear-stressed BAECs. Lanes: 1, 5, and 9, no nuclear extracts; 2, 6, and 10, nuclear extracts from static BAEC; 3, 7, and 11, nuclear extracts from BAECs exposed to 10 dynes/cm² laminar shear stress for 4 h; 4, 8, and 12, addition of the relevant (SS-1, SS-2, or SS-3) unlabeled probe in 100-fold excess. (B) Gel-shift analysis of the complexes formed by the oligonucleotide probe SS-4 with nuclear extracts from static and shear-stressed BAECs. Lanes: 1–3, as in A; 4, addition of unlabeled relevant probe, 100-fold excess; 5, addition of unlabeled Oct-1, 100-fold excess. Sequences of oligonucleotide probes: SS-1, gatTGAAGGGTTGCTCGGCTCTCAGAGACCC; SS-2, gatCTCTCAGAGACCCCTAAGCGCC; SS-3, gatCCCCCTAAGCGCCCCGCCCTGCCCCAAC; SS-4, gatCTCTCAGAGACCGATCATCTCTCAGAGACC. The gat triplet was added to each probe to allow efficient radiolabeling. The putative SSRE sequence is underlined in SS-1, SS-2, and SS-4.

Table 2. Conservation of SSRE core binding sequence in promoters of genes responsive to shear stress

Gene	Origin	Location, nt	Sequence	Ref(s)
PDGF-B	Human	–125	TCTCAGAGACC	20, 21
PDGF-B	Feline	–125	TCTCAGAGACC	23
PDGF-B	Murine	–125	TCTCAGAGACC	24
tPA	Human	–284	TATGGGAGACC	26
tPA	Rodent	–252	CCTTTGAGACC	26
tPA	Murine	–252	CCTTTGAGACC	26
TGF- β 1	Human	–396	ACGTCGGTCTC	27
TGF- β 1	Murine	–1314	GTGGCGAGACC	28
ICAM-1	Human	–649	GTGGTGAGACC	29

Position of the SSRE relative to the initiation of transcription. Note that the core binding sequence (GAGACC) identified in this study and its complementary sequence (GGTCTC) are conserved among the PDGF-B promoters of several species and in unrelated genes that are also responsive to shear stress in vascular endothelium.

element. The regulation of these transcription factors by cytokines, hormones, and other stimuli currently is a topic of intensive investigation (for review, see refs. 48 and 49); it will be instructive to compare the mechanisms coupling these stimuli with those involved in shear-stress-induced gene expression.

In conclusion, the presence of a putative transcriptional factor binding site within the 5' promoter region of several unrelated genes, all of which can respond to shear stress in vascular endothelium, suggests a mechanism for transduction of biomechanical stimuli at the blood-vessel-wall interface. Further molecular characterization of the transcription factor(s) that interact with the SSRE defined in this report and elucidation of their regulation by defined mechanical forces and other stimuli should provide insights into endothelial function in physiological and pathophysiological states.

Note Added in Proof. The SSRE core binding sequence (GAGACC) or its complementary sequence is also present in the promoters of the following endothelial-expressed genes (human) that have been demonstrated to be shear responsive: *c-fos*, *c-jun*, monocyte chemotactic protein (MCP-1) (50, 51).

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